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(54) Title: A SUBSTANTIALLY INSULIN-FREE PROTEIN COMPOSITION, PREPARATION AND USE THEREOF, AND PRODUCTS CONTAINING SAME AND PREPARATION THEREOF			
(57) Abstract <p>The invention relates to a substantially insulin-free protein composition which is characterized in that it is prepared by removing bovine insulin from fat-free protein-containing material originating from cow's milk, such as whey, a whey protein concentrate, fat-free milk or a casein solution. The invention also relates to the preparation and use of said protein composition and to products containing same, such as substantially insulin-free infant formulae and other special nutritive preparations and to the preparation thereof.</p>			

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A SUBSTANTIALLY INSULIN-FREE PROTEIN COMPOSITION, PREPARATION AND USE THEREOF, AND PRODUCTS CONTAINING SAME AND PREPARATION THEREOF

The invention relates to a protein composition and the preparation and use thereof, and to products containing same and the preparation thereof.

- 5 The invention relates particularly to a substantially insulin-free protein composition and the preparation and use thereof, and to products containing same, such as substantially insulin-free infant formulae and other special nutritive preparations, and the preparation thereof.

- 10 Juvenile diabetes, i.e. insulin-dependent diabetes mellitus (IDDM), is a disease caused by destruction of the insulin-producing beta cells in the islets of Langerhans in the pancreas while other cells in the islets remain intact. This disease usually breaks out during childhood at the latest.

- 15 In spite of numerous medical studies, the factors causing juvenile diabetes to break out are not yet exactly known. The current view is, however, that the risk of children developing juvenile diabetes is associated with genes and environmental factors, such as nutrition.

- Several epidemiological studies show among other things that exposure to cow's milk proteins in early infancy increases the risk of contracting juvenile diabetes (Gerstein, Diabetes Care 17 (1994) 13-19). Epidemiological  
20 observations have been used to present several hypotheses concerning mechanisms in which cow's milk proteins could act as diabetogenic factors. It has been observed that the immune response to bovine serum albumin (Karjalainen et al., N. Engl. J. Med. 327 (1992) 302-307), beta lactoglobulin (Vaarala et al., Diabetes 45 (1996) 178-182) or beta casein (Cavallo et al., Lancet 348  
25 (1996) 926-28) could lead to beta cell reactivity, but up to now bovine insulin has not even been considered to be a diabetogenic risk factor.

- Cow's milk is known to normally contain small amounts of bovine insulin. The documented milk insulin content varies depending on the assay method, but e.g. an ELISA method has shown contents of about 50 ng/ml. The  
30 insulin content in milk is at its highest (about 330 ng/ml) immediately after calving, and decreases thereafter reaching its constant level (about 46 ng/ml) within about 7 days after calving (Aranda et al., J. Dairy Sci. 74 (12) (1991) 4320-4325).

- The applicant's studies show that administering conventional cow's  
35 milk-based formula to infants induces the production of antibodies to bovine insulin, such as insulin antibodies. Table 1 shows the contents of IgG-class

antibody contents to bovine insulin for children aged 6 months who were given a conventional cow's milk-based formula (Enfamil®) as a supplement and for children of the same age who were breast-fed only. These antibodies cross react with human insulin.

5

Table 1

IgG-class antibody content to cow's insulin in infants who received a conventional formula up to the age of 6 months and in infants who were only breast-fed up to the age of 6 months. The antibody content is given as absorbency units (OD).

	Median of antibody content (variation range)
Breast-fed infants n=6	0.160 (0.134-0.293)
Formula-fed infants n=8	0.480 (0.213-0.656)

10

Statistically significant difference in antibody contents:  $p=0.04$

15

Since the presence of insulin auto-antibodies (IAA) precedes and presages the development of IDDM, immunization to bovine insulin in cow's milk-based products may be harmful and increase the risk of contracting IDDM. Accordingly, there is a need for commercially available cow's milk products and cow's milk-based products that do not contain immunoreactive bovine insulin.

20

Liquid chromatography and a reversed phase column (Kroeff et al., J. Chromatogr. 461 (1989) 45-61; Poll et al., J. Chromatogr. 539 (1991) 37-45; Cox, J. Chromatogr. 599 (1992) 195-203; Welinder, J. Chromatogr. 542 (1991) 83-99), gel filtration or an anion exchange column (WO 90/00176 and WO 90/00177) or a weak cation exchange column (DE 3511270 A1 and GB 2173503 A) have usually been employed in the purification of insulin from production and extraction liquors. As such, reversed phase or gel filtration chromatography is not suited to treating milk, since the milk treated should be suitable for foodstuffs and reasonably priced.

25

However, none of the prior art publications the applicant is familiar with presents or even suggests the separation or removal of the bovine insulin present in cow's milk.

30

The applicant has discovered how to produce a protein composition suited to foodstuffs inexpensively from cow's milk. The composition is substantially free of bovine insulin and suitable as such for infant formulae in par-

ticular, but even as the protein component of other special nutritive preparations.

Thus the invention relates to a substantially insulin-free protein composition which is characterized in that it is prepared of fat-free protein-containing material originating from cow's milk, such as whey, a whey protein concentrate, fat-free milk or a casein solution.

By removing fat and casein from milk, whey containing the whey proteins is obtained. About 20 percent of the total milk protein are whey proteins and the rest is casein. The whey obtained in cheese production is called cheese whey and the whey obtained in casein production is in turn called casein whey.

The whey used in the invention can be any whey originating from cow's milk, such as cheese whey, rennet casein whey, acidic casein whey or sour cheese whey. The whey is preferably cheese whey.

The fat-free protein-containing material originating from cow's milk can also be a whey protein concentrate.

In addition to the above, the fat-free protein-containing material originating from cow's milk and used in the invention can be fat-free milk or a water solution made of fat-free milk powder or a water solution made of milk casein, i.e. a casein solution.

The substantially insulin-free protein composition of the invention can be suitably prepared by the method of the invention, the method being characterized by

a) passing a liquid fat-free protein-containing material originating from cow's milk to insulin removal treatment, whereby it is passed through a column filled with a strong cation exchange resin regenerated to  $\text{Na}^+$  or  $\text{K}^+$  form, the pH value being 2 to 7, suitably 5.2 to 5.6,

b) the liquid protein-containing material obtained in step a) being concentrated by ultrafiltration and diafiltration by using membranes which are 6,000 to 20,000 D cut-off membranes, evaporating the obtained protein concentrate, and drying it to protein powder,

c) optionally

1) forming of the protein concentrate or protein powder obtained in step b) a solution with a protein content of 1 to 20% in water,

2) subjecting the protein solution obtained in step c1) to enzymatic

hydrolysis with proteases, and

- 3) ultrafiltering the protein solution hydrolyzed in step c2), and
- d) optionally passing the protein hydrolysate obtained in step c) to hydrophobic chromatographic treatment, and
- 5 e) evaporating and drying to powder the solution obtained in step c) or d).

In step a) the liquid fat-free protein-containing material originating from cow's milk is led to insulin removal treatment, whereby substantially all or at least a significant part of the bovine insulin in said material is removed. In  
10 insulin removal said liquid material originating from cow's milk is treated with a strong cation exchange resin. Additionally it can be clarified by e.g. micro filtration or centrifugation. At this stage the whey can also be clarified by ultrafiltration.

However, the best insulin removal results are obtained when the  
15 material to be purified is first clarified as described above and then treated with a strong cation exchange resin.

It is most advantageous to purify whey, such as cheese or casein whey or a diluted whey protein concentrate, of bovine insulin with a strong cation exchange resin. Suitable cation exchange resins include Amberlite C-20  
20 (Rohm & Haas, France) and Spherosil S. (Rhone-Poulenc, France). Other suitable cation exchange resins include Amberlite IR-120 and Finex VO 7 (Finex Oy, Finland).

The whey to be purified whose pH has been adjusted to a value between 2 and 7, suitably to a value between 5.2 and 5.6 with an acid of food-stuff quality, e.g. HCl, or by ion exchange, is passed at 3 to 65°C, suitably at  
25 room temperature, through a column filled with a strong cation exchange resin regenerated to Na<sup>+</sup> or K<sup>+</sup> form. In batch process the feeding rate and volume may vary, but the feeding rate is suitably 1 to 10 column volumes (BV)/h and feeding volume 5 to 60 BV, preferably 20 BV. At the pH range 5.2 to 5.6 whey  
30 proteins are usually negatively charged, but insulin is positively charged since its isoelectric point is 5.6 (Erkama, *Biokemia*, p. 185). Hereby at least a substantial part of the bovine insulin in the whey is bound to the cation exchange resin while the negatively charged whey proteins pass through the column.

The whey is preferably purified of bovine insulin by a strong cation  
35 exchange resin at pH 5.4.

No bovine insulin was found in the whey purified with a strong

cation exchange resin when assayed by electro-spray mass spectrometry.

A corresponding treatment with a strong cation exchange resin can also be used to significantly reduce the bovine insulin content of fat-free cow's milk and an aqueous solution made of milk casein, i.e. a casein solution.

5 Hereby the insulin content of the casein solution decreases at least about 60% and the insulin content of fat-free milk in turn decreases at least about 30%.

Insulin was analyzed from the samples with an electro-spray mass spectrometer (VG Quattro II, VG BioTech, Aitrincham, England) by using fractionation with a C18 reversed phase column of a liquid chromatograph (Hewlett Packard HPLC 1090, Hewlett Packard Co., Germany) (eluent: A: 10  
0.05% trifluoroacetic acid (TFA) in water, B: acetonitrile (Acn) + 0.05% TFA, gradient: 15% → 40% in 20 min, 40% → 100% in 10 min) as a pretreatment. The insulin-containing fraction was freeze dried, dissolved again to a concentrated solution and led to a mass spectrometer. Insulin was also assayed by  
15 RIA analysis (radio immuno assay).

Whey can be purified of bovine insulin by clarifying it by e.g. micro-filtration, ultrafiltration or centrifugation, whereby residual casein and other macromolecular proteins to which the insulin is bound as hydrophobic protein are removed from the whey.

20 In microfiltration the whey to be purified is led suitably at 10 to 60°C through microfiltration membranes which are 0.05 to 1.4 micrometer membranes, preferably 0.1 micrometer membranes.

In ultrafiltration, in turn, the whey to be purified is treated with ultra-filtration membranes which are preferably membranes with a cut-off value of  
25 50,000 to 200,000 Dalton.

In centrifugation the whey is treated preferably at a rate of 1,000 to 10,000 rounds per minute.

Clarification treatment decreases the bovine insulin content of whey by 6 to 10%.

30 Clarification treatment and cation exchange resin treatment both reduce the bovine insulin content of the treated material originating from cow's milk, but the best results are obtained by first clarifying said material and then leading it to cation exchange resin treatment. The best results are obtained in whey treatment, wherein the most preferable clarification procedure is micro-  
35 filtration.

In step a) the protein-containing material, preferably whey, purified

of bovine insulin, is concentrated by ultrafiltration and diafiltration to obtain a sufficient protein content, and the obtained protein concentrate is then evaporated and optionally dried to protein powder. Hereby the material, at least significantly purified of bovine insulin, such as whey, whose pH is adjusted to a value of about 6.5 using a base of foodstuff quality, e.g. NaOH or  $\text{Ca}(\text{OH})_2$ , or by ion exchange, is led to ultrafiltration and diafiltration, whereby, by using a semi-permeable membrane, proteins having a heavier molecular weight are separated from lactose, salts and proteins with a lighter molecular weight, such as insulin whose molecular weight is about 5734 D. The cut-off value of a semi-permeable membrane is suitably 6,000 to 20,000 D, preferably 10,000 D, and e.g. a polyether sulphone membrane with a cut-off value of 10,000 D can be used as the semi-permeable membrane.

The whey obtained in step a) can be concentrated using a total concentration ratio which is suitably about 120. Hereby the whey treated in step a) is ultrafiltered preferably by 10,000 D cut-off membranes, first e.g. at a concentration ration of 10, and the retentate is then diluted to starting volume and refiltered at a ratio of 12, resulting in a total concentration ratio of 120. The obtained whey protein concentrate whose protein content is about 90% of dry matter is evaporated and dried to powder by e.g. spray drying or frost drying.

The whey protein powder obtained by using conventional ultrafiltration and diafiltration and then drying and having a protein content of 70 to 80% usually contains 43 to 48 micrograms insulin per gram of powder (about 60 micrograms insulin per gram of protein).

In contrast, the whey protein powder obtained in accordance with steps a) and b) of the method according to the invention contains significantly less bovine insulin than the above conventionally ultrafiltered and diafiltered whey protein powder. By an electro-spray mass spectrometer, no bovine insulin at all was found in the whey protein powder made of the whey purified of bovine insulin with by a strong cation exchange resin in step a). The obtained substantially insulin-free protein composition is suitable as such for use as a raw material in e.g. infant formulae and other special nutritive preparations since the whey protein it contains is nutritionally of very high quality and does not need other proteins to complete the nutrient content.

Cation exchange resin treatment can also be performed on e.g. fat-free milk or an advantageous protein preparation, milk casein, as an aqueous solution. The bovine insulin residue can be removed from the casein solution



in the same way as from milk. Because its nutritive value is less than that of whey protein, casein is not, however, as recommendable as the exclusive protein source for infant formulae as is whey protein.

If the aim is to reduce the bovine insulin content of the protein composition of the invention still further, enzymatic hydrolysis, and optionally even hydrophobic chromatographic treatment can be added to its preparation. In this case a solution with a content of 1 to 20%, preferably about 5%, is formed in water of the protein concentrate or protein powder obtained in step b). The pH of the solution is adjusted to about 8.5 with e.g.  $\text{Ca}(\text{OH})_2$  and the temperature to about 50°C, and animal or microbial enzymes are then added to the solution in such a way that they efficiently hydrolyze bovine insulin protein chain linkages in particular. Such proteases include chymotrypsin, subtilisin Carlsberg serine protease, subtilisin BPN' serine protease, serine and metalloproteases of *Aspergillus oryzae*, papain, *Bacillus subtilis* neutral protease, thermolysin, serine and metalloproteases of *Streptomyces griseus*, pepsin, acid protease of *Endothica parasitica* and pancreatin. The hydrolysis is allowed to continue for 8 hours. In the hydrolysis bovine insulin is split into peptides of the length of at most five amino acids. These peptides do not cause immune response nor contain epitopes contained in the previous insulin molecule. To remove undegraded macromolecules, the obtained hydrolysis mixture is led to ultrafiltration through a dense ultrafiltration membrane, which is suitably a 2,000 D cut-off membrane. The obtained permeate is dried to powder by e.g. spray drying. No bovine insulin is found in the obtained product.

If the intention is to remove bovine insulin hydrolysis products from the mixture as completely as possible, the above obtained whey protein hydrolysate can be led suitably as a 10% solution to hydrophobic chromatographic treatment for removal of hydrophobic peptides and therewith possible bovine insulin hydrolysis products from the hydrolysate.

Said chromatographic treatment can be carried out suitably using a hydrophobic adsorption resin, such as Amberlite XAD-16 resin (Rohm & Haas, France), but it is also possible to use activated charcoal which is also able to remove hydrophobic compounds. In practice said chromatographic treatment can be carried out in production scale in a column packed with adsorption resin or activated charcoal through which the solution to be treated is passed. The solution that has passed the column is recovered, evaporated and dried to powder. The powder obtained does not contain bovine insulin in quantities that

are observable by current methods. As far as hydrophobic amino acids are concerned, the amino acid composition of the product has, however, changed somewhat and thus small amounts of phenylalanine and tyrosine has to be added during the production of nutritive preparations.

5           Bovine insulin is preferably removed from fat-free protein-containing material originating from cow's milk, preferably whey, by a strong cation exchange resin, and the treated liquid protein-containing material is then concentrated by ultrafiltration and diafiltration. As clarification treatment, microfil-  
10       tration is advantageous since insulin is often bound to macromolecules, such as casein dust or denatured whey protein which can be suitably removed by microfiltration. If the above methods do not produce a sufficiently low bovine insulin content in the product, the content can be further lowered by means of enzymatic hydrolysis and optionally hydrophobic chromatography in association therewith.

15           Infant formulae usually contain milk, cream, vegetable oil, low-salt whey powder, minerals and vitamins, of which milk, cream and low-salt whey powder contain bovine insulin. Whey protein has a very high nutritional value and is therefore suitable for the single protein source in infant formulae and other special nutritive preparations, too.

20           The substantially insulin-free protein composition of the invention can be used as the protein component of infant formulae and other special nutritive preparations and e.g. as the raw material of consumption milk. This provides a product which is free of bovine insulin and does not cause immunization to bovine insulin nor increase the risk of contracting IDDM.

25           Thus the invention also relates to a substantially insulin-free infant formula and a substantially insulin-free special nutritive preparation, characterized in that the substantially insulin-free protein component therein is made of fat-free protein-containing material originating from cow's milk, such as whey, a whey protein concentrate, fat-free milk or a casein solution, preferably,  
30       however, of whey, suitably in the above described manner.

          The invention will be described in greater detail in the following examples.

#### Example 1

35           The pH of 6,000 ml fresh cheese whey was lowered with 10% HCl to 5.4. 300 ml of strong cation exchange resin (Amberlite C-20, Rohm & Haas, France) were packed into a 300 ml laboratory scale column and regenerated

with 600 ml of 17% NaCl. The column was then rinsed with 1,000 ml water. 6,000 ml cheese whey (20 column volumes (BV)) were run through the column at room temperature at a rate of 6 BV/h. According to electro-spray mass spectrometric analysis, before treatment the whey contained 343 ng/ml insulin, i.e. 68 mg insulin/kg real protein. After treatment no insulin at all was found in the whey, i.e. the treatment decreased the content by 100%. The total protein content of the whey did not significantly change during the treatment. Before treatment the protein content was 0.87%, after treatment 0.86%. The pH was raised with 10% NaOH again to 6.5, and the whey was then ultrafiltered by 10,000 D cut-off membranes, first at a concentration ratio of 10, then the retentate was diluted to starting volume and refiltered at a ratio of 12, resulting in a total concentration ratio of 120. The protein concentrate was freeze dried to a powder containing 90% protein.

#### Example 2

100 litres of cheese whey filtered through 0.1  $\mu$ m microfiltration membranes are ultrafiltered by 10,000 D cut-off membranes first at a concentration ratio of 10, then the retentate is diluted to starting volume and filtered again at a ratio of 12, resulting in a total concentration ratio of 120. The retentate contained protein 90% of dry matter and it was spray dried to powder (drying temperatures 180/75°C). The untreated cheese whey contained 343 ng/ml insulin, i.e. 68 mg/kg of real protein. The microfiltered whey contained 324 ng/ml insulin, i.e. 64 mg/kg of real protein. According to a mass spectrometer, the insulin content of the ultrafiltered and diafiltered whey protein concentrate was 21 mg/kg real protein, i.e. the content decreased in proportion to the protein about 69%. Conventional ultrafiltered whey protein powder containing 70 to 80% protein contains insulin about 60 mg/kg of real protein. According to RIA analysis, the untreated cheese whey contained insulin in proportion to protein 178  $\mu$ IU/g of protein, and the treated, ultrafiltered and freeze dried powder 100  $\mu$ IU/g of protein. Thus the removal ratio was 44%.

#### Example 3

100 litres of acid whey obtained from preparation of acid casein were microfiltered similarly through 0.1  $\mu$ m membranes. The pH of the whey was 4.5. The microfiltered whey was ultrafiltered and diafiltered as in example 1 so as to obtain a total concentration ratio of 120. The protein content was then about 90% of dry matter. The untreated whey contained insulin 320 ng/ml, i.e. 48 mg/kg real protein, the microfiltered whey 295 ng/ml, i.e. 45

mg/kg real protein, and the ultrafiltered and diafiltered whey protein concentrate contained insulin 95 ng/ml, i.e. 16 mg/kg real protein when assayed by mass spectrometry. Thus the insulin content decreased about 67% in the treatment.

5           Example 4

Into 60 litres of water at 50°C were dissolved 5.040 kg of the whey protein powder prepared in example 1, 11.423 kg vegetable fat mixture, 11.232 kg glucose, 12.260 kg maltodextrin (DE 21), 135 g vitamin and mineral pre-mixture (containing A, D, E, K, B1, B2, B6, B12 vitamins, niacin, folic acid, 10 pantothenic acid, biotin, ascorbic acid, choline, inositol, ferrous gluconate, zinc sulphate, manganese sulphate, sodium selenite, copper gluconate), and 70 g calcium chloride, 300 g calcium phosphate, 65 g magnesium sulphate, 125 g sodium chloride and 620 g potassium citrate. The dry matter content of the mixture was about 40%. The obtained mixture was led to a homogenizer 15 (150/50 bar) and dried to powder by a spray drier at drying temperatures of 180/75°C, on a fluidized bed 70/120/30°C. The composition, appearance and taste of the product were equal to those of a conventional infant formula powder.

Example 5

20           The whey protein concentrate presented in example 1 was diluted to a 5% content with water. The solution was pasteurized at 65°C for 20 min and cooled to 50°C. The pH was adjusted to 8.5 with 10% Ca(OH)<sub>2</sub>. 6% of the protein quantity of pancreatin enzymes (4 x USP, SPL, USA) and Alcalase 0.6 L enzymes (Novo Industri A/S, Denmark) were added to the mixture. During 25 hydrolysis the pH of the mixture was kept at 7.0 by additions of Ca(OH)<sub>2</sub>. The mixture was allowed to hydrolyze for 8 hours and then the mixture was annealed for 5 min at 90°C. The mixture was then cooled to 50°C and ultrafiltered with 2,000 D cut-off membranes, and the permeate was recovered. The obtained permeate was spray dried to powder. No insulin could be found in the 30 hydrolysate.

Example 6

35           The hydrolysate obtained in example 5 was dissolved to a 10% solution. 30 ml Amberlite XAD-16 resin (Rohm & Haas) were packed into a laboratory-scale column which was regenerated with 60 ml of 4% NaOH, rinsed with 1,000 ml water and regenerated with 60 ml of 4% HCl and rinsed with water until the pH of the water passing it exceeded 5. The hydrolysate solution

was run through a 1,700 ml resin column, corresponding to 567 g of hydrolysate dry matter/100 ml resin. The passed solution was recovered and freeze dried to powder. No insulin was found in the hydrolysate. The hydrolysate was used as the single protein source of a special nutritive preparation intended for people allergic to milk.

#### Example 7

In 60 litres of water at 50°C were dissolved 8.670 kg of the whey protein hydrolysate powder prepared in accordance with example 6, 10.466 kg vegetable fat mixture, 16.058 kg glucose, 5.233 kg maltodextrin (DE 21), 135 g vitamin and mineral mixture (containing A, D, E, K, B1, B2, B6, B12 vitamins, niacin, folic acid, pantothenic acid, biotin, ascorbic acid, choline, inositol, ferrous gluconate, zinc sulphate, manganese sulphate, sodium selenite, copper gluconate), and 10 g calcium chloride, 320 g calcium phosphate, 70 g magnesium sulphate, 165 g sodium chloride and 620 g potassium citrate, 1 g L-tyrosine and 2 g L-phenylalanine. The dry matter content of the mixture was about 40%. The obtained mixture was led to a homogenizer (150/50 bar) and dried to powder by a spray drier at drying temperatures of 180/75°C, on a fluidized bed 70/120/30°C. The composition, appearance and taste of the product were identical to those of a conventional special nutritive preparation intended e.g. for people allergic to milk.

#### Example 8

A strong cation exchange column (30 ml resin) was regenerated as in example 1. The pH of 600 ml fat-free milk was adjusted to 5.4. The pH-adjusted milk was run at room temperature through the column as in example 1, whereby some calcium, but also a significant part of the insulin was removed from the milk. According to RIA analysis (no mass spectrometer was available for milk) the untreated milk contained bovine insulin 26 µIU/ml and the treated one 17 µIU/ml. The treatment decreased the insulin content about 35%. The treatment was insufficient for total removal of insulin from the milk, but the example shows that the method is also suited to the treatment of other milk raw materials than whey.

#### Example 9

A cation exchange resin (30 ml) was regenerated as in example 1. A 3% solution in water was prepared of sodium caseinate. The pH was lowered to 5.5 with dilute HCl. The caseinate solution was pumped through the cation exchange resin at room temperature as the milk in example 8. Accord-

ing to RIA analysis, the untreated caseinate solution contained insulin 26  $\mu$ U/ml and the treated solution 10  $\mu$ U/ml. The insulin content decreased about 62% in the treatment.

#### Example 10

5 Insulin removal assay was carried out by an ultrafiltered whey protein concentrate containing 35% protein of dry matter. The whey protein concentrate was diluted with water to a 5.1% solution, the pH was adjusted from 6.2 to 5.3 with 20% HCl. A strong cation exchange resin Amberlite C-20 (30 ml) was regenerated with 60 ml of 17% NaCl and rinsed with water. The resin  
10 was packed into a column and 600 ml (20 BV) whey protein concentrate were run through the column at a rate of 180 ml/h. According to RIA analysis the whey protein concentrate contained 11.7  $\mu$ U/ml insulin before treatment, the protein content being 1.95%. After treatment the insulin content was 4.8  $\mu$ U/ml and the protein content the same, 1.95%. The treatment decreased the  
15 insulin content by 59% with no change in the protein content.

#### Example 11

The assay was repeated with an ultrafiltered whey protein concentrate (35% protein of dry matter) which had been microfiltered through 0.8  $\mu$ m membranes. The whey protein concentrate was diluted with water to a 5.1%  
20 solution, the pH was adjusted with 20% HCl from 6.3 to 5.4. A strong cation exchange resin Amberlite C-20 (30 ml) was regenerated with 60 ml of 17% NaCl and rinsed with water. The resin was packed into a column and 600 ml (20 BV) whey protein concentrate were run through the column at a rate of 180 ml/h. According to RIA analysis, the whey protein concentrate contained  
25 11  $\mu$ U/ml insulin before treatment, the protein content being 1.93%. After treatment the insulin content was 2.25  $\mu$ U/ml and the protein content 1.90%. The treatment decreased the insulin content 79% and the protein content only 1.5%. The example shows the advantageous effect of microfiltration on insulin removal.

#### Example 12

30 Insulin removal from whey was assayed using two different types of strong cation exchange resins Finex VO 7 and Amberlite IR-120. The assays were carried out as in example 11, except that the pH values were 4.0, 5.5, 5.8 and 6.4. At pH 4.0 the IR-120 resin removed 16% of the insulin, whereas  
35 at pH 5.5 and 6.4 it did not remove any insulin. At pH 4 the VO 7 resin removed 7.5% of the insulin whereas at pH 5.8 and 6.4 it did not remove any

insulin. The resins were not efficient insulin eliminators but their effect increased as the pH fell. The example shows that at least for some resins the acid range is a more advantageous range than the neutral range. Protein loss in the different assays was only 3 to 6%.

## CLAIMS

1. A substantially insulin-free protein composition, **characterized** in that it is prepared by removing bovine insulin from fat-free protein-containing material originating from cow's milk, such as whey, a whey protein concentrate, fat-free milk or a casein solution.

2. A method of preparing a substantially insulin-free protein composition, **characterized** by

a) passing a liquid fat-free protein-containing material originating from cow's milk to insulin removal treatment, whereby it is passed through a column filled with a strong cation exchange resin regenerated to Na<sup>+</sup> or K<sup>+</sup> form, the pH value being 2 to 7, suitably 5.2 to 5.6,

b) the liquid protein-containing material obtained in step a) being concentrated by ultrafiltration and diafiltration by using membranes which are 6,000 to 20,000 D cut-off membranes,

evaporating the obtained protein concentrate, and optionally drying it to protein powder,

c) optionally

1) forming of the protein concentrate or protein powder obtained in step b) a solution with a protein content of 1 to 20% in water,

2) subjecting the protein solution obtained in step c1) to enzymatic hydrolysis with proteases, and

3) ultrafiltering the protein solution hydrolyzed in step c2), and

d) optionally passing the protein hydrolysate obtained in step c) to hydrophobic chromatographic treatment, and

e) evaporating and drying to powder the solution obtained in step c) or d).

3. A method as claimed in claim 2, **characterized** in that whey, a whey protein concentrate, fat-free milk or a casein solution, preferably whey, is used as the liquid fat-free protein-containing material in step a).

4. A method as claimed in claim 2 or 3, **characterized** in that in step a) the liquid fat-free protein-containing material is clarified before cation exchange resin treatment by filtering it through microfiltration membranes, the membranes being 0.05 to 1.4 micrometer membranes, preferably 0.1 micrometer membranes.

5. A method as claimed in claim 2 or 3, **characterized** in



that in step a) the liquid fat-free protein-containing material is clarified before cation exchange resin treatment by treating it with ultrafiltration membranes, which are preferably 50,000 to 200,000 D cut-off membranes.

5 6. A method as claimed in claim 2 or 3, **characterized** in that in step a) the liquid fat-free protein-containing material is clarified before cation exchange resin treatment by centrifuging it preferably at a rate of 1,000 to 10,000 rounds per minute.

7. A method as claimed in any one of claims 2 to 6, **characterized** in that membranes which are 10,000 D cut-off membranes are  
10 used in the ultrafiltration and diafiltration in step b).

8. A method as claimed in any one of claims 2 to 7, **characterized** in that an aqueous solution with a content of 1 to 20%, preferably about 5%, is formed of the protein concentrate ultrafiltered and diafiltered in step b), the aqueous solution being subjected to enzymatic hydrolysis with  
15 proteases, such as pancreatin enzymes and alcalase enzymes.

9. A method as claimed in claim 8, **characterized** in that the protein hydrolysate obtained in step c) is led to hydrophobic chromatographic treatment, whereby it is treated with activated charcoal or preferably with a hydrophobic adsorption resin.

20 10. A protein composition as claimed in claim 1, **characterized** in that it is prepared by a method according to any one of claims 2 to 9.

11. The use of a protein composition according to claim 1 or 10 as the protein component of an infant formula or other special nutritive preparation, or as the raw material of consumption milk.

25 12. A substantially insulin-free infant formula, **characterized** in that its protein component is prepared of a substantially bovine insulin-free fat-free protein-containing material originating from cow's milk, such as whey, a whey protein concentrate, fat-free milk or a casein solution, preferably whey.

30 13. A substantially insulin-free special nutritive preparation, **characterized** in that its protein component is prepared of a substantially bovine insulin-free fat-free protein-containing material originating from cow's milk, such as whey, a whey protein concentrate, fat-free milk or a casein solution, preferably whey.

35 14. A method of preparing a substantially insulin-free infant formula or other special nutritive preparation or consumption milk or a raw material therefor, **characterized** in that a protein composition prepared accord-

ing to any one of claims 2 to 9 is used as its protein component.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 98/00369

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A23J 1/20, A23C 9/146

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A23J, A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, MEDLINE, BIOSIS, EMBASE, CA, FSTA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Dialog Information Services, file 155, Medline, Dialog Accession No. 09441450, Medline Accession No. 98156374, Vaarala O. et al: "Cow milk feeding induces antibodies to insulin in children a link between cow milk and insulin-dependent diabetes mellitus", Scand J Immunol (ENGLAND) Feb 1998, 47 (2) p131-5	1-14
	--	
A	Dialog Information Services, file 155, Medline, Dialog Accession no. 08868938, Medline Accession no. 97033794, Vahasalo P. et al: "Relation between antibodies to islet cell antigens, other autoantigens and cow's milk proteins in diabetic children and unaffected siblings at the clinical manifestation of IDDM. The childhood Diabetes in Finland Study", Autoimmunity (SWITZERLAND) 1996, 23 (3) p165-74	1-14
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

### \* Special categories of cited documents:

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- "E" earlier document but published on or after the international filing date
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Date of the actual completion of the international search

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 98/00369

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>EP 0601802 A1 (VALIO LTD.), 15 June 1994 (15.06.94)</p> <p style="text-align: center;">-- -----</p>	1-14

Information on patent family members

PCT/FI 98/00369

Form PCT/ISA/210 (patent family annex) (July 1992)

